THE DUAL ROLE OF PYRUVATE AND THE ENERGY REQUIREMENT IN NITROGEN FIXATION

R. W. F. Hardy and A. J. D'Eustachio

Central Research Department*, E. I. du Pont de Nemours and Company, Experimental Station, Wilmington, Delaware

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The optimum fixation of elemental nitrogen by cell-free extracts of Clostridium pasteurianum requires high levels of either α-ketobutyrate or pyruvate (0.11 M) (Carnahan, et al., 1960), while the efficiency of this reaction is only 3-6%. These observations suggest that in addition to furnishing reducing power, pyruvate has another role. The major pathway of pyruvate metabolism in this organism is a phosphoroclastic reaction, and a steady-state level of acetyl coenzyme A, acetyl phosphate and ATP is produced. McNarry and Burris (1962) have shown inhibition of N_2-fixation with arsenate or glucose and suggested that high-energy phosphate is required for fixation. However, Carnahan, et al. (1960) and McNarry and Burris (1962) found that addition of acetyl phosphate or ATP inhibited rather than stimulated nitrogen fixation. Mortenson (1963) attributes the glucose inhibition to depletion of inorganic phosphate and suggests that high-energy phosphate may not be required for nitrogen fixation. The involvement of activated phosphate in nitrogen fixation is demonstrated in the present report, and for the first time pyruvate has been replaced by the combination of an ATP-generator and a reductant, KBH_4.

METHODS

Cell-free extracts were prepared by autolysis of dried cells as previously described by Carnahan, et al. (1960). Modification of the cell-growing procedure included a decrease in phosphate content of the medium to 0.53 mM (8% of the previous level) and the use of a pH-stat in place of $CaCO_3$.

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Nitrogen-fixing activity was measured as net ammonia synthesis using the modified micro-Conway method of Mortenson (1961); in some cases it was confirmed by N_2^{15} assay according to the method of Burris and Wilson (1957). Acetyl phosphate was measured as acethydroxamate according to Lipmann and Tuttle (1945); pyruvate was determined by the 2, 4-dinitrophenylhydrazone method of Friedemann and Haugen (1943).

RESULTS AND DISCUSSION

Nitrogen fixation is inhibited almost completely by arsenate (0. 05 M) or glucose (0. 063 M) plus hexokinase, but not by glucose alone. For ammonia synthesis pyruvate cannot be replaced by reductants such as 0. 0125-0. 1 M KBH_4, 0. 0125-0. 1 $Na_2S_2O_4$, or 0. 4 atm. of H_2. Although time-course studies of N_2-fixation showed a strong dependence of ammonia production on pyruvate degradation, as much as 0. 06 μmoles of NH_3/mg protein are formed during the 12 min. period immediately following the exhaustion of pyruvate (Figure 1). This ammonia synthesis in the absence of pyruvate parallels a decrease in the level of acetyl phosphate from 0. 59-0. 00 μmoles/mg protein. These three indirect lines of evidence suggest that in addition to a suitable electron donor, activated phosphate is required for N_2-fixation.

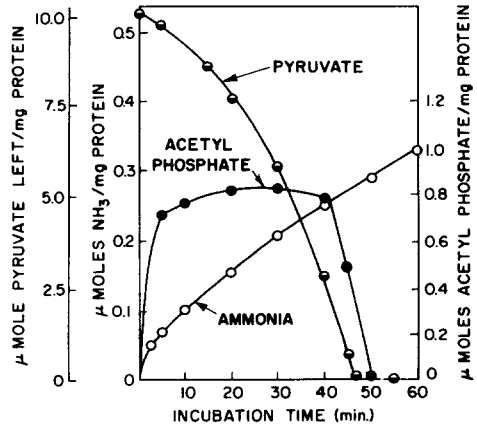

Figure 1. Time-course of N_2-fixation and pyruvate degradation.

The reaction flask contained 3. 64 mmoles of pyruvate, 0. 4 moles KH_2PO_4, pH 6. 5, 1. 6 moles cacodylate, 0. 35 g of protein extract and water to 35. 0 ml under a N_2 atmosphere. The flask was stirred at 26°C and samples automatically withdrawn and assayed every 2 minutes.

The addition of creatine phosphate (0.025 M) plus creatine kinase to an incubation mixture containing an optimum level of pyruvate (0.11 M) increased ammonia synthesis by 50% while added acetyl phosphate (0.0125-0.050 M) had little effect (Table 1). Both ATP-generators stimulated N_2-fixation, by an incubation mixture containing a limiting level of pyruvate (0.023 M). The stimulation by 0.025 M acetyl phosphate was 4.3-fold.

TABLE 1

Stimulation of N_2-fixation by Pyruvate with ATP-generators

Pyruvate	ATP-Generator	mM ATP-Generator added 0	12.5	25	50
M		mμmoles NH_3 formed/mg protein/min.			
0.11	Acetyl phosphate	5.61	5.53	4.22	4.87
0.11	Creatine phosphate	6.78	8.07	10.20	9.60
0.023	Acetyl phosphate	0.34	-	1.82	-
0.023	Creatine phosphate	0.34	-	0.76	-

Incubation mixtures contained in 4 ml: cell-free extract protein, 44 mg; pyruvate as indicated; KH_2PO_4, pH 6.5, 150 μmoles; ADP, pH 6.5, 1 μmole; dilithium acetyl phosphate as indicated; disodium creatine phosphate as indicated; and creatine kinase, 1 mg when creatine phosphate was present and they were covered by 0.8 atm. of argon for the control or 0.8 atm. of N_2 for the experimental.

Substantial ammonia synthesis occurred when pyruvate was replaced with dilithium acetyl phosphate (0.05 M) and potassium borohydride (0.01 M); this activity has been confirmed by N_2^{15} assay. The KBH_4 was found to be most effective when added in two portions. One part, called "pre-addition" KBH_4, was added to the pre-cooled incubation flask just prior to flushing and covering with argon for the control or N_2 for the experimental. The other part, "substrate" KBH_4, was placed in the side-arm along with acetyl phosphate, both in the dry state. After flushing with argon or N_2 these compounds were tipped into the enzyme solution just prior to incubation. Typical ammonia synthesis with the acetyl phosphate-borohydride system is shown in Table 2. The complete system shows ammonia formation 50-100% of that customarily obtained

with pyruvate, while omission of the enzyme extract or dilithium acetyl phosphate or "substrate" KBH_4 resulted in no ammonia synthesis. The omission of "pre-addition" KBH_4 substantially reduced N_2-fixation. However, significant ammonia synthesis was not produced by "pre-addition" borohydride in the absence of "substrate" KBH_4. The optimum levels of "substrate" KBH_4, "pre-addition" KBH_4, dilithium acetyl phosphate and crude enzyme extract are those recorded in Table 2. Total fixation decreases as the amount of "substrate" borohydride decreases but even 0.001 M KBH_4 produced 1.0 mμmoles NH_3/mg protein/min. In the presence of creatine kinase, acetyl phosphate is replaceable with an equal amount (0.05 M) of creatine phosphate.

TABLE 2

Replacement of Pyruvate with
Potassium Borohydride and Acetyl Phosphate

Component Omitted	NH_3 formed mμmoles/mg protein/min.
None	4.70
Cell-free extract	.17
Dilithium acetyl phosphate	00
"Substrate" KBH_4	.07
"Pre-addition" KBH_4	3.03

Complete incubation system contained in 4 ml: crude enzyme extract, 44 mg protein; "pre-addition" KBH_4, 1 mg; "substrate" KBH_4, 2 mg; dilithium acetyl phosphate, 200 μmoles; KH_2PO_4, pH 6.5, 100 μmoles, and was covered by 0.8 atm. of argon for the control or 0.8 atm. of N_2 for the experimental.

An ADP dependence for the acetyl phosphate-borohydride system was demonstrated. Dialysis of a crude extract against 0.05 M pH 6.5 phosphate buffer for 2 hours at room temperature under 0.8 atm. of H_2 reduced its activity for ammonia synthesis from 3.51 mμmoles/mg protein/min. to 0.67, while addition of ADP (0.625 mM) to the dialyzed preparation restored its activity to 3.87.

Thus the dual role of pyruvate in nitrogen fixation as an energy source and a reducing agent has been indirectly demonstrated by inhibition and addition experiments and directly demonstrated by replacement experiments. The stimulation of ammonia synthesis by the

addition of ATP-generators to limiting levels of pyruvate suggests that the high pyruvate requirement is due to energy demands. The ability of creatine phosphate plus creatine kinase to replace acetyl phosphate and the requirement of ADP to reactivate a dialyzed preparation suggests that ATP is the required metabolite for energy donation. It is proposed, on the basis of rate studies, that ATP directly or indirectly activates the enzyme rather than a coenzyme and that this activated enzyme combines with N_2 and uses its energy derived from the ATP to activate the stable nitrogen molecule for reduction to ammonia.

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